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Initial Characterization of the Two ClpP protease isoforms from Chlamydia trachomatis

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I. Acknowledgements

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II. Introduction

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that is the causative agent of sexually transmitted infections (STI) and the ocular disease known as trachoma (CDC.gov). Trachoma is the leading cause of preventable blindness worldwide and most heavily affects developing nations (WHO.org). *C. trachomatis* causes over 1.5 million STIs each year in the U.S. alone and is of particular concern to women as 70–80% of genital tract infections are asymptomatic and 15–40% ascend to the upper genital tract, which can lead to serious diseases including pelvic inflammatory disease, infertility, and ectopic pregnancy[2]. *C. trachomatis* STIs have also been shown to facilitate the transmission of HIV and are associated with increased risk of cervical cancer in HPV-infected women[2]. Although chlamydial infections are currently treatable with antibiotics, drug resistance has been observed *in vitro,* treatment failure occurs clinically (although the underlying cause of failure remains unclear), and chlamydial reservoirs in the intestine are not susceptible to drug concentrations that are effective in clearing genital tract infections. In addition, a vaccine has not been developed[3]. Collectively, the large numbers of infections and sub-optimal methods available for treatment and prevention suggest that alternative approaches are needed for combatting *C. trachomatis*.

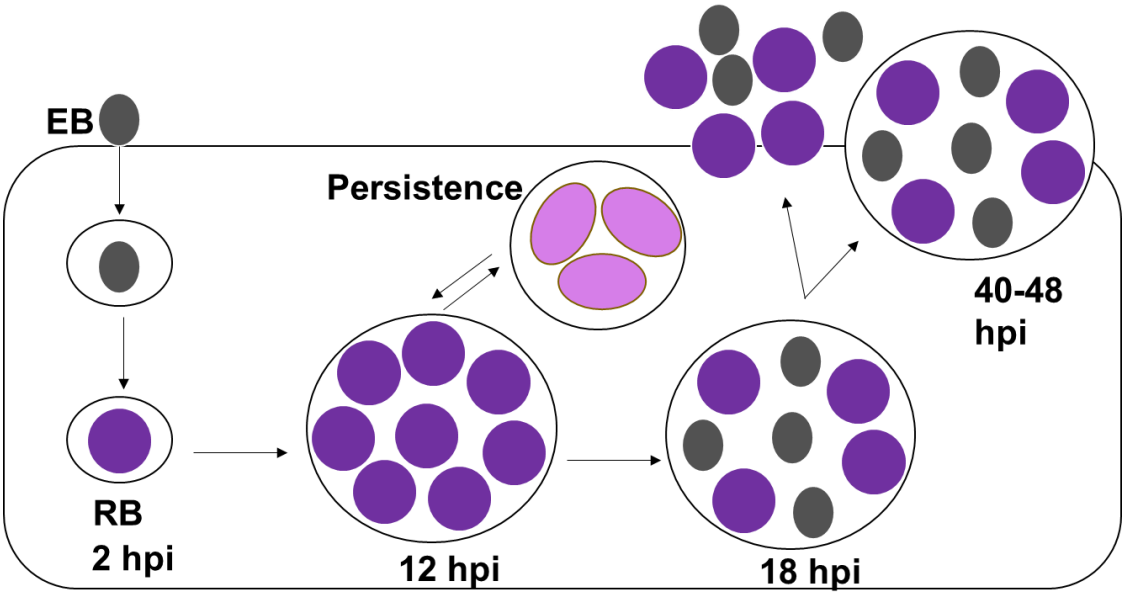

Figure 1. The biphasic developmental cycle of *Chlamydia*. *Chlamydia* differentiates between the infectious elementary body (EB) and the replicative reticulate body (RB). *Chlamydia* begins the infection in the EB form before differentiating into the RB to replicate within an inclusion; after multiple replications, the RBs will asynchronously differentiate back into EBs before exiting the cell. Under stress conditions, *Chlamydia* will enter a form of low activity termed persistence. Times are given in terms of hours post infection (hpi).

C. trachomatis has been shown to undergo a unique biphasic developmental cycle in which it differentiates between two functionally distinct forms[5] (Fig 1). At the initial time of infection, *C. trachomatis* exists in its infectious form known as the elementary body, or EB. The EB is an electron dense structure that initiates cellular-uptake and creates a vacuole out of the host cell's membrane termed an inclusion. Proteins required for central metabolism and glucose catabolism were more prevalent in the EB form, suggesting that a basal metabolism is required outside of the cell to facilitate EB maintenance and cellular invasion[4]. Within the inclusion, *C. trachomatis* will then differentiate into the reticulate body (RB) form which is characterized as a larger and less dense structure that replicates within the inclusion. In the RB form, *C. trachomatis* synthesized more proteins associated with protein synthesis, ATP transport, and nutrient transport suggesting that the RB form is more focused on utilizing available nutrients, increasing cellular mass, and obtaining resources to divide and eventually differentiate back into the EB form[4].

The different forms and "life-styles" of the EB and RB necessitate differences in gene activation and protein content. Multiple transcriptomic and proteomic studies have documented these differences. Consequently, *Chlamydia* need a mechanism to aid in proteome turn-over during differentiation. Additionally, and conserved amongst other organisms, *Chlamydia* also require a system for degrading proteins that are mis-folded, damaged, or no-longer needed. While mechanisms carrying out both limited and global protein degradation would appear to be essential for chlamydial growth and development, making them excellent therapeutic targets, it remains unknown how protein degradation occurs in these highly significant pathogens.

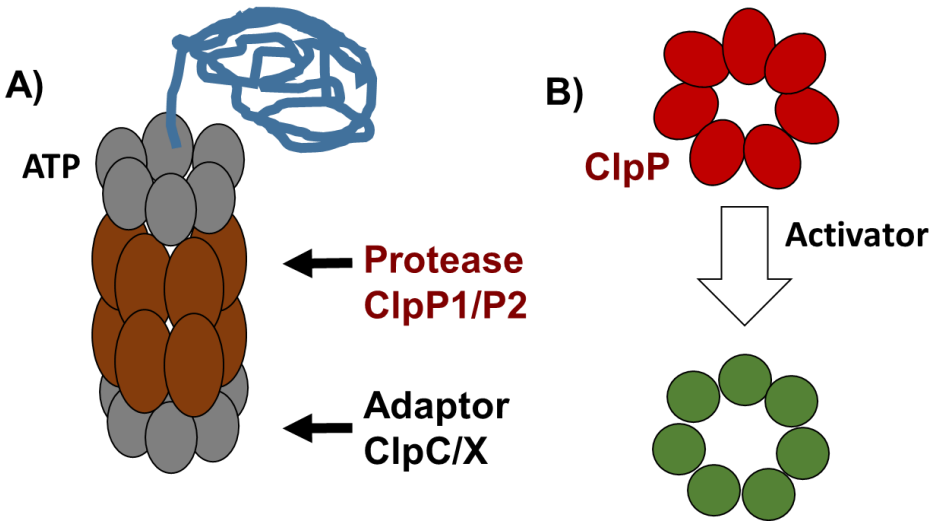

Figure 2 The Clp system. The Clp proteins will form homo-oligomers of heptamers (ClpP1/P2) and hexamers (ClpC/X) which will then oligomerize together (A). While the proteases can function without ATP, the adaptors are ATP-dependent. These protease heptamers are normally seen with a narrow opening, but the activating compounds can aid in opening up the barrel (B).

The Clp protease system is a conserved protein complex that is responsible for regulating proteomic turnover in bacteria and some eukaryotes (Fig 2). This system is comprised of two main parts: the proteasome complex comprised of ClpP (the "trash-compactor") and various adaptor proteins (the "trash-collectors") that target proteins to and help to feed proteins into the proteasome. All chlamydial genomes appear to have components of the Clp system based on annotation and we hypothesize that the Clp system is essential for chlamydial development and therefore infection. The protease complex is comprised of the ClpP protein(s) (ClpP1 and ClpP2 in *Chlamydia*) while the adaptors for *Chlamydia* are ClpC and ClpX. These proteins are non-functional in their single form, meaning one ClpP protein has no protease activity. However, when it oligomerizes with other ClpPs they form a barrel unit comprised of seven proteins. This heptameric barrel will then oligomerize on top of a second barrel to create the active dodecatetramer form. In this double-barrel form, the ClpP proteasome can only degrade small peptides and a limited number of unfolded protein substrates as the opening at the top of the double barrel is relatively narrow. As a method of regulation, the adaptor protein will open the barrel and selectively target proteins to the proteosome. ClpC and ClpX will each form a hexameric barrel, which will then bind to the top and bottom of the ClpP double barrel and unfold proteins to feed them into the barrel. In *Bacillus subtilis*, it has been shown that arginine phosphorylation via McsA/McsB will target specific proteins for degradation, similar to ubiquitination in eukaryotes, which the adaptor proteins will respond to and only open the proteasome for those targeted proteins[10].

As the *Chlamydia* genome is small (1.039 megabases) due to reductive evolution during its life-span as an obligate, intracellular bacterium, it is expected that it only encodes for those proteins absolutely necessary for survival[6]. For this reason, it is interesting to note that *Chlamydia* encode two paralogs of the ClpP protein, ClpP1 and ClpP2. This protein is a well characterized protease in other organisms; however, in most other organisms, such as *Escherichia coli*, there is only one ClpP. In the organisms that encode two ClpPs they have been shown to interact with each other, such as in *Mycobacteria*[7]. As the Clp proteins have been shown to interact with each other in other organisms it is possible they might in *Chlamydia* as well; however, it is also possible they preform distinct functions as shown in *Pseudomonas*[9].

The increased prevalence of antimicrobial resistant strains across clinically-relevant bacterial species has led to a frantic search for new antimicrobials and antimicrobial targets. The essentiality of the Clp system based on genetic studies in a number of bacteria makes it an attractive target for drug development. ClpP activating compounds have been identified that will open up the ClpP proteasome to cause unregulated degradation of proteins in other organisms[7]. These activating compounds are the acyldepsipeptides (ADEP) and activators of cylindrical proteases (ACP) and they have distinct mechanisms of action. In *Bacillus subtilis* the ADEP compounds were crystalized in their bound form to the ClpP proteins and it was discovered they bind to the H pocket of the formed oligomers, hypothetically mimicking the binding of the adaptor proteins, and open the double barrel[11]. ACPs, in comparison, have been seen to bind at two different regions, the H pocket and the C pocket, and are hypothesized to

steady the formation of the double barrel that is expected for the ClpP proteins[11]. Both approaches lead to the same desired result – lethality for the bacterium.

For this study, we hypothesized that the Clp system plays an essential role in the proteomic turnover needed for differentiation between the EB and RB, and therefore is required for the developmental cycle. As a first test of our hypothesis we sought to determine if the ClpPs were functional using various *in vitro* approaches and whether ClpP functionality is required for chlamydial growth. The latter point was tested using a cell-culture infection model with various ACPs. We determined that 1) ClpP2, but not ClpP1, appears to be active *in vitro* under the conditions tested, 2) that ClpP2 is expressed during development, and 3) that ClpP2 activating compounds severely inhibit chlamydial growth in tissue culture.

III. Results

We initially sought to determine if the *clp* genes were expressed in *C. trachomatis.* This work was done by Scot Ouette's group (data not shown) and corroborated by our lab by looking at protein production through Western blot analysis. To detect protein expression, we took advantage of a prior study on *Pseudomonas aeruginosa*, in which antibodies were synthesized against PA1801 (*P. aeruginosa* ClpP1) and PA3326 (*P. aeruginosa* ClpP2). The antibody against *P. aeruginosa* ClpP1 showed amino acid sequence similarity to *C. trachomatis* ClpP2 and it was confirmed through western blot analysis that the antibody would bind ClpP2 (Fig 3A). Using this data, it was found that ClpP2 is expressed in *C. trachomatis* at 24, 30, and 40 hours post infection (Fig 3B) when RBs would be converting to EBs.

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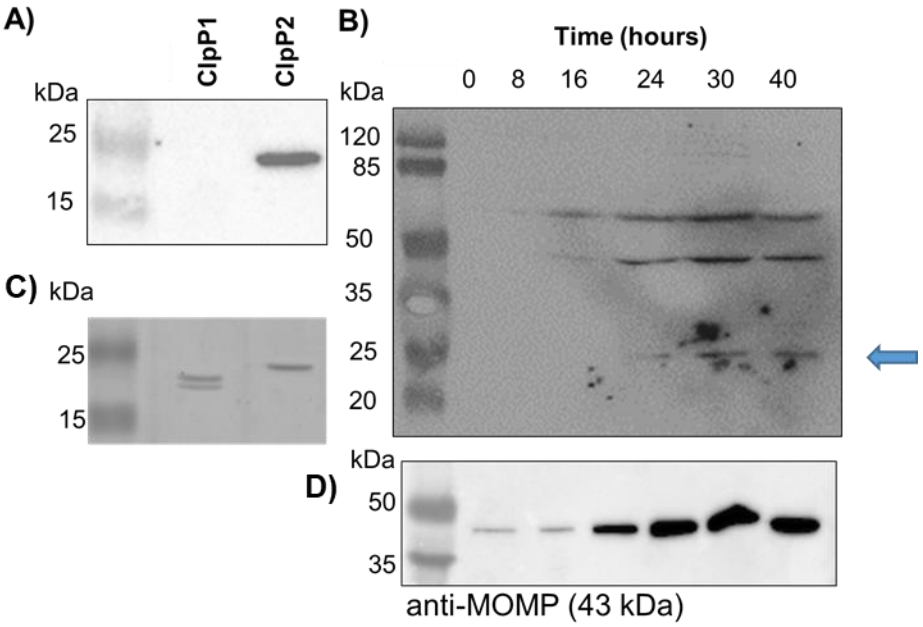


Figure 3 Western blot analysis of ClpP2 expression in *C. trachomatis*. Purified proteins were run on 12% SDS-PAGE gels and then transferred to nitrocellulose for anti-ClpP2 tag western blot (A and B). Coomassie brilliant blue (C) and anti-MOMP western blot (D) controls were also run. Expected molecular weights for the recombinant proteins: ClpP1 22 kDa and ClpP2 23 kDa,

Escherichia coli BL21(de3) was used to produce the respective ClpP proteins using the *lac* operator and T7-promoter regulated pLATE31 vector (Fig 4). Each Clp protein gene was cloned into the multiple cloning site of the pLATE31 vector using ligation independent cloning.

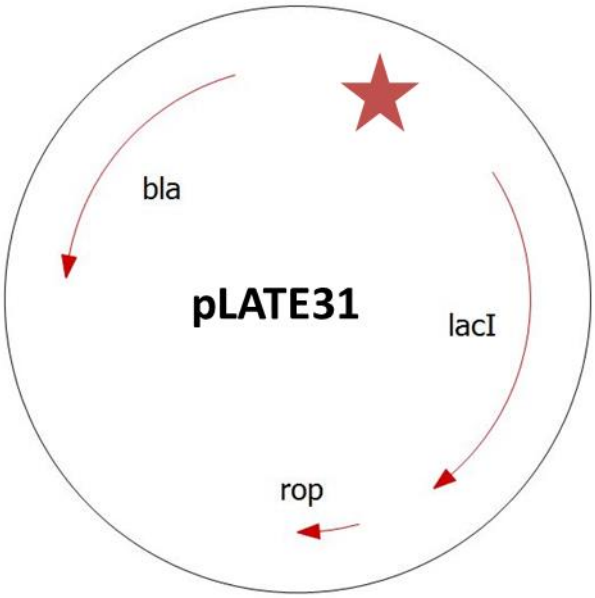


Figure 4 Clp protein producing plasmids. The *clpP1* (*ctl0690*), *clpP2* (*ctl0075*), *clpP1SDM*, and *clpPec* genes were cloned into pLATE31 at the multiple cloning site (starred region) to produce C-terminal His-tagged recombinant proteins under control of the *lac* operator and T7 RNA polymerase promoter

The proteins were then affinity purified using cobalt-based immobilized metal affinity chromatography. The ClpP1 product appeared to have two proteins at different sizes (Fig 5) as seen by the double band. It was determined through mass spectrometry that the smaller band protein sequence began at a second methionine located within the *ctl0690* gene, while the larger band had the predicted native start site methionine (Fig 5). To address this potential problem as the N-terminal sequence is crucial for oligomerization, a mutant of the ClpP1 protein was created where the second and third internal methionines were replaced with an isoleucine and leucine, respectively. This mutant was called ClpP1SDM. ClpP2 did not express this double band and was therefore utilized in its native form. As a positive control, *E. coli* ClpP was expressed and purified for assays (ClpPec). Purifications of ClpP1(SDM) and ClpP2 were completed multiple times.

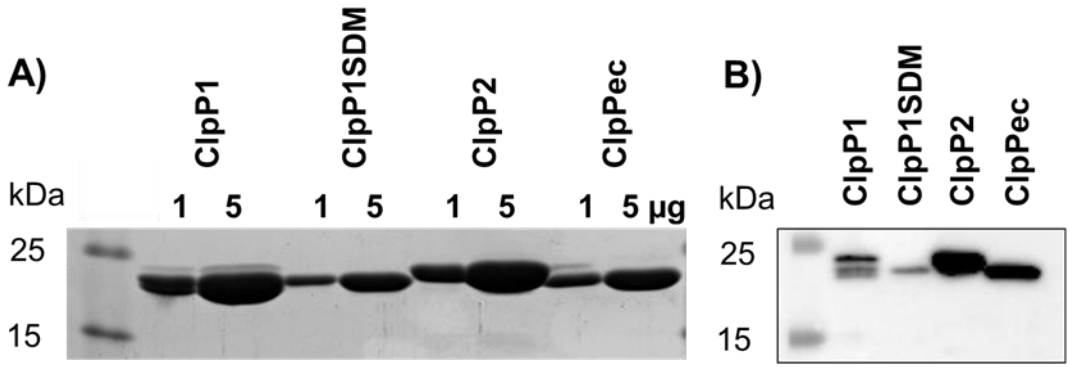


Figure 5 Purification of recombinant ClpP1, ClpP1SDM, ClpP2, and ClpPec. Purified proteins were run on 12% SDS-PAGE gels and then either stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose for anti-His tag western blot (B). Expected molecular weights for the recombinant proteins: ClpP1 22 kDa, ClpP1SDM 22 kDa, ClpP2 23 kDa, ClpPec 24 kDa.

Activity was assessed for ClpP1, ClpP2, ClpP1SDM, and ClpPec using an oligomerization assay, digestion of a small fluorescent peptide, and digestion of casein in the presence or absence of potential activating compounds. Oligomerization assays were performed using native-PAGE gels and demonstrated that ClpPec formed the strongest dodecatetramer, while ClpP2 and ClpP1 formed primarily the heptamer with modest dodecatetramer, while ClpP1SDM appears to only form the heptamer (Fig 6). These results suggest an *in vitro* functionality ranking of ClpPec being more active than ClpP1 and ClpP2, which are about equal, and those are more active then ClpP1SDM. To more directly address protease activity further experimentation was performed.

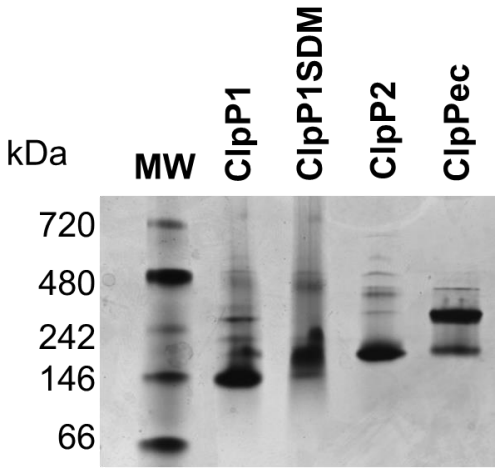
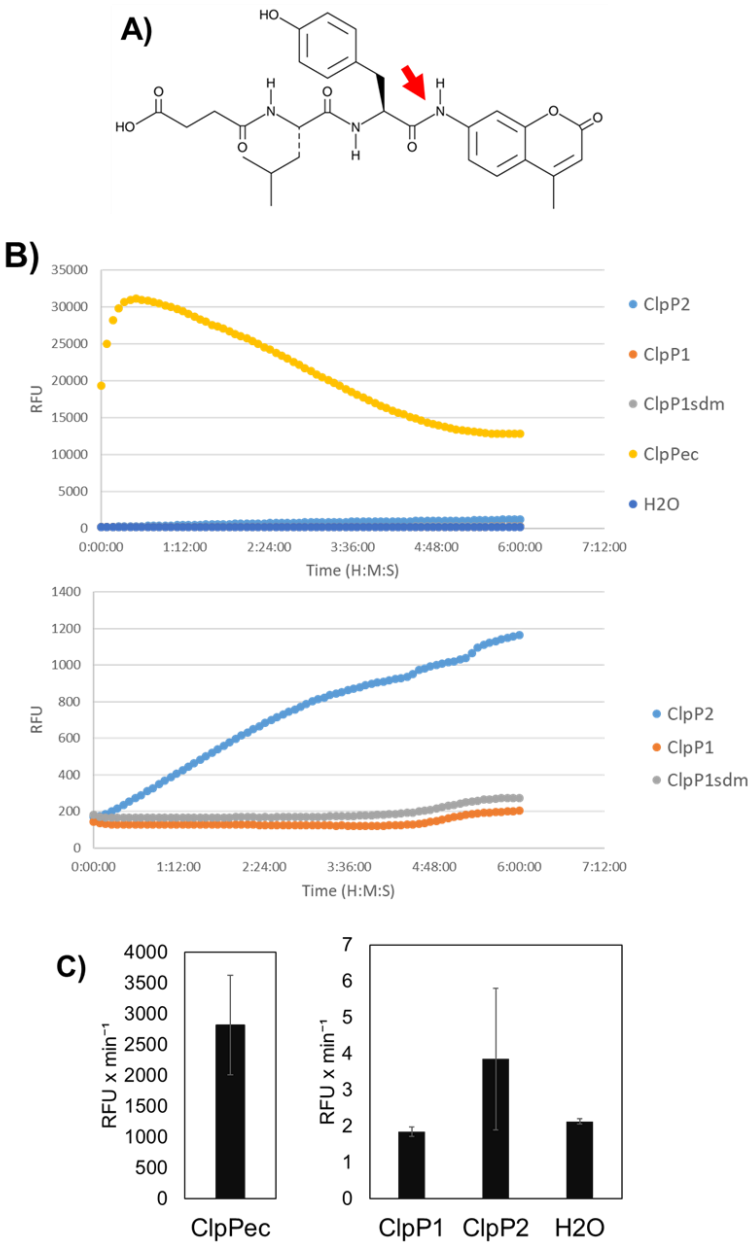


Figure 6 The ClpP proteins form oligomers. Purified proteins were run on a Native-PAGE gels and then stained with Coomassie Brilliant Blue. Expected molecular weights for the heptamer and dodecatetramer oligomers: ClpP1 154/308 kDa, ClpP1SDM 154/308 kDa, ClpP2 161/322 kDa, ClpPec 168/336 kDa.

Activity was initially assayed using a fluorescent substrate, Suc-Leu-Tyr-ACM, which when cleaved releases AMC to fluoresce (Fig 7A). The fluorescence readout is directly proportional to the level of ClpP activity and readings were taken over six hours. It was seen that while ClpPec was very active throughout the assay, ClpP1 and ClpP1SDM were both found to be inactive, and ClpP2 had detectable, but modest activity (Fig 7B). There was a two-fold increase in fluorescence between ClpP1 (at baseline) and ClpP2 (Fig 7C).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ClpP1sdm
ClpPec
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RFU x min⁻¹
ClpPec
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1
0
RFU x min⁻¹
ClpP1
ClpP2
H2O

Figure 7 ClpP2 is active *in vitro* while ClpP1 is not. The ClpPs (1 µM) were incubated with the fluorometric peptide Suc-Luc-Tyr-AMC at 37°C for 6 hours. The peptide fluoresces when cleaved at the site indicated (A). Relative fluorescence units were recorded at five-minute intervals and the average of all time course traces are shown in (B). The average values are reported with standard error in (C, n = 3).

An *in vitro* assay supported these findings, in which β-Casein was used to test the protease activity of the ClpP analogs with and without the activating compounds. β-Casein is a much larger protein than Suc-Leu-Tyr-AMC, and the ClpP analogs should be unable to degrade it without an adaptor protein to unfold the substrate or an activating compound to increase the barrel opening. It was found that ClpPec, ClpP1, and ClpP2 were all unable to degrade β-casein. However, upon the addition of 10 µM of ADEP1, and 500 µM of the ACP1, ACP1a, and ACP1b β-casein was mostly degraded by ClpPec and ClpP2 after 3 hours (Fig 8).

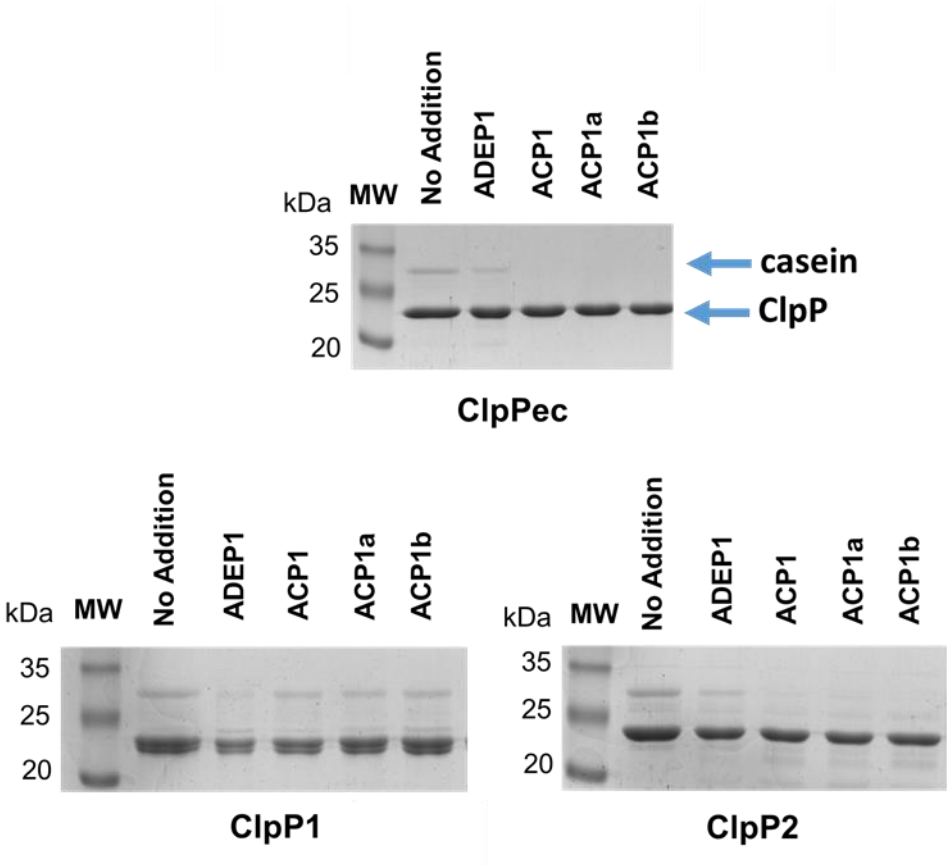

Figure 8 ACP and ADEP activate ClpP2, but not ClpP1. Reactions were run at 37°C for 3 hours with 1 µg of casein. ADEP1 was used at 10 µM and other compounds were used at 500 µM. Representative gels are shown.

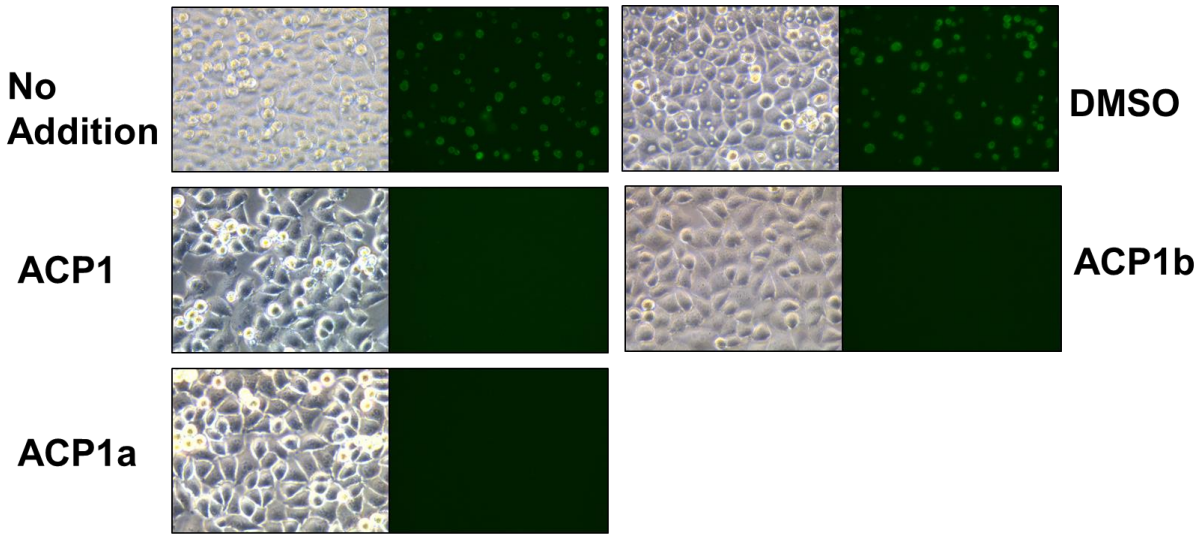

Figure 9 ClpP activating compounds inhibit *C. trachomatis* growth. *In vitro* results were corroborated with an *in vivo* assay. HeLa cells were infected with *C. trachomatis* at a MOI of 0.2 and activators were used at a concentration of 50 µg/mL. Wells were imaged at 24 and 48 hours post infection (24 shown). No bacteria were present in the presence of inhibitors at 48 hours post infection as well.

After demonstrating that: 1) ClpP2 is expressed and the protein could be detected during growth, 2) ClpP2 oligomerizes, 3) ClpP2 can cleave a peptide substrate, and that 4) ClpP2 activity is stimulated towards casein using ACP and ADEP, we sought to test whether ClpP2 activation would disrupt chlamydial growth. Compounds were previously identified as antibacterial like compounds in other organisms specifically targeting the bacterial Clp system. These activating compounds, known as acydepsipeptides (ADEP compounds) and activators of cylindrical proteases (ACP compounds), have been seen in *E. coli* to enhance ClpP activity. ADEP1, ACP1, ACP1a, and ACP1b were seen *in vivo* to enhance *C. trachomatis* Clp system activity (ADEP1 not pictured) (Fig 9). *C. trachomatis* strain DFCT28 has been modified to express GFP. Infected cells were treated with 50 µg/mL of each specified activating compound in duplicate and the chlamydial growth was inhibited in all wells. The untreated infected cells and DMSO treated infected cells showed no change in growth (Fig 9).

IV. Discussion

In this study, we sought to biochemically characterize the *C. trachomatis* proteins ClpP1 and ClpP2 with the goal of validating the Clp system of *Chlamydia* as a therapeutic target. Our data is indicative of the Clp system playing an essential role in the development of *Chlamydia,* as it is expressed at specific time points in the development cycle and unregulated activity caused chlamydial cell death.

This study began by testing Clp protein expression in *C. trachomatis* to determine at what point in the developmental cycle the Clp proteins may regulate proteomic turnover. To do so, an antibody was identified as specific for *Pseudomonas aeruginosa* ClpP1 and *C. trachomatis* ClpP2. When a western blot was run using infected L2 cells at varying points in the developmental cycle, ClpP2 was detected at 24, 30, and 40 hours post infection. This correlates to the differentiation from RB back to EB at about 24 hours post infection up until the release of EBs from the host at about 40 hours post infection. This correlates well with our hypothesis that the Clp system regulates proteomic turnover from one developmental form to the next as it is initially expressed at the specific time point in the developmental cycle known for that exact transition. However, ClpP2 expression is lacking at the initial transition from elementary to reticulate body, though this is not entirely surprising as we are open to the idea that the elementary body form comes prepackaged with all the proteins and nutrients needed to make this single transition. It is also possible that ClpP1 is critical for early steps, but it may require an adaptor protein for functionality (such as ClpX/C) that has yet to be identified.

As discussed earlier, ClpP proteins are not active until they are in their dodecatetramer double barrel. From the oligomerization assays it became clear that the purified ClpP proteins from *C. trachomatis* did not readily oligomerize, while those from *E. coli* did. The *E. coli* band was significantly stronger and this could be the result of expressing the protein, ClpPec, in its original organism, which would easily lend to proper folding as it is in its ideal environment. Chlamydial proteins, on the other hand, were not in their ideal environment for expression, as they were expressed in *E. coli,* rather than *C. trachomatis*. Soluble protein was purified and some oligomerized forms of the protein could be seen, but they were stronger at the expected size for heptamers rather than the dodecatetramers. This does not mean that the proteins are inactive, however, as the fluorescence and Casein assays are indicative of ClpP2 activity, simply that with the current expression system the proteins may be less active than they would be in their native form. For this reason, it is important to note our *in vivo* studies that show a low amount of activating compound is still enough to cause *Chlamydia* cell death. In their native environment, ClpP activity may be stronger than our *in vitro* studies have shown with the current models.

While we initially saw ClpP2 can readily degrade a small fluorescent peptide, ClpP1 was unable to do so. We hypothesized this may be due to the two ClpP1 proteins that were identified on an SDS-PAGE gel and verified by mass spectrometry. This led us to create a mutant of ClpP1 through site directed mutagenesis to replace the internal

methionines with an isoleucine and leucine, creating ClpP1SDM. When this mutant was tested in the same fluorescence assay, it did not show the same level of activity as ClpP2. One purification did show a high level of activity, but later multiple purifications did not, causing us to conclude the initial purification may have been contaminated with ClpPec. From this it appears that ClpP1 may need an additional protein/peptide for proper function, or the isoleucine and leucine rendered it inactive and a different mutant needs to be created.

This finding was further supported by the β-Casein assay that found ClpP2, with the assistance of activating compounds, could degrade β-Casein while ClpP1 could not. This lack of activity is indicative of there either not being a fully formed ClpP1 double barrel, which is supported by the oligomerization assay, a mix of proper ClpP1 proteins and those lacking 4 amino acids, which might lead to inactivity, or ClpP1 is simply inactive in *Chlamydia.* It is unlikely that ClpP1 has no activity considering the chlamydial genome is so small, as previously discussed, but still possible.

The *in vivo* assay is incredibly important to this study as it exemplifies the importance of the Clp system in *Chlamydia's* developmental cycle, based on the clearance of the infection upon the addition of the activating compounds. Also, as seen in the non-GFP images, the cells that had the infection cleared look healthy and normal, supporting the idea that while the activating compounds are non-specific among bacterial species, they might not target the hosts' Clp system or inhibit cells under the conditions tested.

From this study, we were unable to determine any interaction between the ClpP1 and ClpP2 proteins nor determine if there is any specificity between each ClpP analog and the ClpC/X adaptor proteins. This is a source for further experimentation and study as well as the determination of more specific activating compounds. While the *in vivo* assay supported that the hosts Clp system was unaffected by the activating compounds there are still beneficial microbiota in multicellular organisms that an ideal treatment would not target. Other activating compounds have already been identified, but they did not exhibit species specificity and had a lessened effect on the *Chlamydia in vivo*.

Based upon findings from this study, the Clp system appears functional in *Chlamydia* and our *in vitro* and *in vivo* results support the Clp system as a novel target for drug development.

V. Methods

Cloning of the ClpP genes

Table 1: Primers used to isolate genes. The primers were designed using Clone Manager.

Primers Used		
Gene	Forward Primer	Reverse Primer
ctl0075	AGAAGGAGATATAACTATGACGTT GGTACCATACGTTGTTGAAG	GTGGTGGTGATGGTGATGGCCAG ACGCAATACTCTTATCTTTTGTC
ctl0690	AGAAGGAGATATAACTATGCCTGA AGGGGAAATGATGCATAAG	GTGGTGGTGATGGTGATGGCCCAA GTCGTTAAAAGAGAAGAGAATCCC
ctl0690 mutant	ATAGAAAGTTGTTGGATTCTCGTC G	CTATGACATCTTGCAACTTATGAAT CAGTTCCCCTTCAGGCATAGTTATA TC
eck0431	AGAAGGAGATATAACTATGTCATAC AGCGGCGAACGAG	GTGGTGGTGATGGTGATGGCCATT ACGATGGGTCAGAATCGAATCG

The *clpP1* (*ctl0690*), *clpP2* (ctl*0075*), *clpP1SDM* and *clpPec* genes were amplified through PCR using the primers designated in the above table and inserted into the vector pLATE31using the LIC procedure as described by the Fisher Scientific (Kit #K1261). The primers were designed using Clone Manager and the completed vectors were then transformed into *Escherichia coli* NEB10 competent cells through chemical transformation and transformants were selected on LB agar plates supplemented with 100 µg/ml ampicillin. Clones were assessed for the gene inserts using PCR and the plasmid was then extracted using the GeneJet Plasmid purification kit (Fisher Scientific #K0502). The plasmid was then sequence verified and transformed into *Escherichia coli* BL21 (de3) through electroporation for protein expression.

Protein Expression and Purification

The ClpP constructs were induced at 0.5mM IPTG at 18°C for 20 hours in *E. coli* BL21 (de3). Both proteins are fused to a His tag and were purified using immobilized metal affinity chromatography with HisPur Cobalt Agarose resin (Fisher Scientific). The culture was grown in 500mL of lysogeny broth with ampicillin to an OD_{600nm} reading of 0.6. After induction the sample was centrifuged at 13,000g for 20 minutes, washed in PBS, and frozen overnight. The pellet, after thawing, was then resuspended in a 1:10-1:12.5 solution of Buffer A. The cells were lysed through sonication in five, ten second bursts. The sample was again centrifuged at 13,000g for 20 minutes. The soluble portion was then filter sterilized into a bed of 200µL of resin. The protein was bound to the resin for 1 hour, after which, the resin was spun down at 700g for 5 minutes at 4°C and the supernatant was then discarded. The resin was washed with 400µL of Buffer A, centrifuged at 700g for 1 minute, and the A_{280} value was read until it leveled out. Then 200µL of Buffer B was added to the resin, mixed, and centrifuged at 700g for 5 minutes.

This was repeated three more times. Each step was run on an SDS-PAGE gel to confirm protein purification and elution, and then the protein yield was concentrated by pooling all of the elution samples in an Amicon unit, adding 10mL of Buffer C, and centrifuging it at 4,000g for 90 minutes. This was repeated two more times. The final protein preparation was stored at -80°C and protein concentrations were determined by a Bradford Assay.

Buffer compositions:
Buffer A: 25mM Tris (pH 7.5), 150mM NaCl, 10mM Imidazole, 10% glycerol
Buffer B: 25mM Tris (pH 7.5), 150mM NaCl, 300mM Imidazole, 10% glycerol
Buffer C: 25mM Tris (pH 7.5), 150mM NaCl, 10% glycerol

Western blots: *Pseudomonas aeruginosa* ClpP1 and ClpP2 antibody comparison and His-tag detection

The *P. aeruginosa* antibodies were rabbit poly-clonal antibodies and were used at the following concentrations: α-ClpP1 antibodies were used at a concentration of 1:10,000, while α-ClpP2 antibodies were used at a concentration of 1:5,000. A test was run of α-ClpP2 antibodies with 1:2,500 with no change in results. α-His antibodies were mouse monoclonal and were used at a 1:2,500 concentration.

The western blot to test for antibody compatibility was run at 300mA for an hour for the transfer of 200ng of each specified protein to nitrocellulose. The blot was blocked using milk protein (casein) for 1 hour at room temperature, the primary antibody was applied overnight at 4°C, and the secondary antibody was applied for 1 hour at room temperature.

The only differences that were applied to the time point western blot were that the transfer was run at 325mA and only 50ng of ClpP2 protein were used. For each time point, 25µl of *C. trachomatis* protein sample were used, 12µl of L2 lysate and 5µl of HeLa lysate were used.

Oligomerization Reactions

Table 2: Expected Protein Sizes of Oligomers. Protein sizes were calculated using the bioinformatics.org website and then multiplied by 7 or 14.

Protein Sizes			
Protein	Weight (with tag)	Oligomer	Weight
ClpP1	22kDa	Heptamer	154kDa
		Tetradecamer	308kDa
ClpP1SDM	22kDa	Heptamer	154kDa
		Tetradecamer	308kDa
ClpP2	23kDa	Heptamer	161kDa
		Tetradecamer	322kDa
ClpPec	24kDa	Heptamer	168kDa
		Tetradecamer	336kDa

The oligomerization reacts were set up to run at room temperature for 40 minutes in Buffer D supplemented with either nothing, 1mM of ATP, or 1mM AMP-PNP. Each reaction had 2µg of protein. The reactions were then mixed with native-PAGE buffer and run on a 4-20% native-PAGE gel at 200 volts for 90 minutes. The final gel was stained with Coomassie brilliant blue.

The second and third biological replicates, run with another batch of purified protein were incubated only in Buffer D but for 1 hour at 37°C with 5µg of protein. This replicate also tested the efficacy of the ClpP activators: ACP1 and ACP1a.

Buffer compositions:
Buffer D: 25mM Tris-HCl (pH 7.5), 5mM KCl, 5mM MgCl2, 10% glycerol, 1mM DTT

Fluorescent Peptidase Assay

These experiments were conducted by incubating 1µM of ClpP protein with 500µM of peptide Suc-Luc-Tyr-AMC in Buffer E. Papain was used as a positive control at 0.1µM. Each experiment was a 50µL reaction that was run in duplicate on a 96 well plate and measured for fluorescence with an excitation at 340/360nm and a reading at 440/460nm. The experiment was conducted over 6 hours at 37°C with readings taken every 5 minutes.

Buffer compositions:
Buffer E: 50mM Tris-HCl (pH8), 200mM KCl, 1mM DTT

Casein Degradation Assay

Reactions were set up with 1µM of ClpP, 1µg of casein per time point, Buffer E and a specified amount of activator in an Eppendorf tube. The reaction was carried out at 37°C for 3 hours. The reaction was then immediately mixed with 2x Laemmli buffer and boiled at 90-100°C for 10 minutes. Each time point was then run on a 12% SDS-PAGE gel for visualization. Activators were prepared by Dr. Conda-Sheridan at the University of Nebraska Medical Center

Buffer compositions:
Buffer E was modified to include casein at a concentration of 1µg/µL

***In vivo* antimicrobial assay**

Mouse L2 cells were infected with GFP expressing *C. trachomatis* at a multiplicity of infection of 0.2. Eight hours after being infected, wells were treated in duplicate with 50µg/mL of the activating compound. The inclusions were then imaged 24 hours post infection using fluorescence microscopy.

VI. References

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